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# Biochemical and genetic analyses of the U5, U6, and U4/U6•U5 small nuclear ribonucleoproteins from *Saccharomyces cerevisiae*

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## ABSTRACT

**We have purified the yeast U5 and U6 pre-mRNA splicing small nuclear ribonucleoproteins (snRNPs) by affinity chromatography and analyzed the associated polypeptides by mass spectrometry. The yeast U5 snRNP is composed of the two variants of U5 snRNA, six U5-specific proteins and the 7 proteins of the canonical Sm core. The U6 snRNP is composed of the U6 snRNA, Prp24, and the 7 Sm-Like (LSM) proteins. Surprisingly, the yeast DEAD-box helicase-like protein Prp28 is stably associated with the U5 snRNP, yet is absent from the purified U4/U6•U5 snRNP. A novel yeast U5 and four novel yeast U4/U6•U5 snRNP polypeptides were characterized by genetic and biochemical means to demonstrate their involvement in the pre-mRNA splicing reaction. We also show that, unlike the human tri-snRNP, the yeast tri-snRNP dissociated upon addition of ATP or dATP.**

**Keywords: affinity chromatography; mass spectrometry; spliceosome assembly**

## INTRODUCTION

Eukaryotic pre-mRNA undergoes several modifications such as pre-mRNA splicing, polyadenylate tail addition, and cap methylation before it is exported from the nucleus to the cytoplasm where it is translated into protein (Moore et al., 1993). The machinery that splices eukaryotic pre-mRNA is termed the spliceosome, a large ribonucleoprotein composed of the pre-mRNA, the U1, U2, and U4/U6•U5 snRNPs, which contain dozens of snRNP-associated factors, and several non-snRNP-associated protein factors (Will et al., 1993; Staley & Guthrie, 1998). Assembly of the spliceosome is believed to first involve a stable interaction of the U1 snRNP with the pre-mRNA (Ruby & Abelson, 1988; Séraphin et al., 1988), followed by the stable association of U2 snRNP (Cheng & Abelson, 1987), and subsequently an interaction of the U4/U6•U5 snRNP (Cheng & Abelson, 1987). The catalytic core of the spliceosome is contained within the U2, U5, and U6 snRNPs, as the U1 and U4 snRNPs are not required for catalytic activity (Yean & Lin, 1991).

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The complexity of the pre-mRNA splicing reaction requires that several snRNAs and dozens of protein factors act at strictly defined and regulated places and times. Our understanding of these requirements continues to advance with the discovery of new factors that participate in the pre-mRNA splicing reaction. Determining novel functionalities in splicing factors is key to our understanding of the myriad interactions that must occur to effect a reaction that would fail if it were misaligned by a single nucleotide. To this end, we embarked on a project to identify novel snRNP-associated factors in yeast to study their role in splicing and to better understand all of the functionalities present in the spliceosome.

Previously, we showed the yeast U4/U6•U5 snRNP contains at least 28 associated proteins. Half of the U4/U6•U5 snRNP proteins are those of the Sm class, which associate with the U4 and U5 snRNAs in the tri-snRNP (SmB, SmD1, SmD2, SmD3, SmE, SmF, and SmG; Gottschalk et al., 1999; Stevens & Abelson, 1999) or the Sm-Like (LSM) class, which associate with the U6 snRNA (Lsm2–Lsm8; Gottschalk et al., 1999; Mayes et al., 1999; Salgado-Garrido et al., 1999; Stevens & Abelson, 1999). Four novel proteins were discovered in the biochemically isolated tri-snRNP, termed Snu13, Snu23, Snu66, and Dib1. The *Schizosaccharomyces*

*pombe* Dib1 homolog was previously identified as a protein involved in the cell cycle (Berry & Gould, 1997). Many of the genes identified that play a role in *S. pombe* splicing confer cell cycle defects upon mutation that may result from the inability to splice the pre-mRNA of genes required for the progression of the cell cycle. Database analysis found several very highly conserved homologs of Snu13 as well as conserved regions of the Snu23 and Snu66 proteins, and demonstrated similarity between the Dib1 protein and yeast thioredoxins (Reuter et al., 1999). In this article, we demonstrate genetic and biochemical evidence for the participation of Snu13, Snu23, Snu66, and Dib1 in the splicing of yeast pre-mRNA.

Previous studies of yeast splicing extract have shown that there exist discrete U5, U6, and U4/U6 snRNPs (Madhani et al., 1990). These snRNPs are likely to be intermediates in the assembly and/or disassembly of the spliceosome; we can therefore divine important information on the assembly of snRNPs and splicing complexes by studying the composition of these snRNPs and the functions of the proteins contained in them. The discrete U4 snRNP is not an abundant species in yeast splicing extracts indicating that it behaves as a limiting factor in tri-snRNP and spliceosome assembly. In this work, we describe the relatively abundant U5 and U6 snRNPs as they exist in yeast splicing extracts and discuss their components prior to their assembly into the U4/U6•U5 snRNP.

The purified U5 snRNP contains 13 proteins: the 7 Sm core proteins and the U5-specific proteins identified in this study, Prp8, Brr2, Snu114, Prp28, Snu40, and Dib1. The novel Snu40 protein is not essential, nor is it present in the U4/U6•U5 snRNP, indicating it may be involved only in the assembly of the U5 snRNP or in tri-snRNP biogenesis. Purified U6 snRNP contains eight U6 snRNP-specific proteins, Prp24 (Shannon & Guthrie, 1991), and a heptameric core of LSM proteins (Mayes et al., 1999; Vidal et al., 1999). Additionally, we demonstrate that, unlike the human tri snRNP, the yeast tri-snRNP, but not the U5 snRNP, undergoes an ATP-dependent structural rearrangement that disintegrates the tri-snRNP into discrete U4, U5, and U6 snRNPs as demonstrated by glycerol gradient analysis.

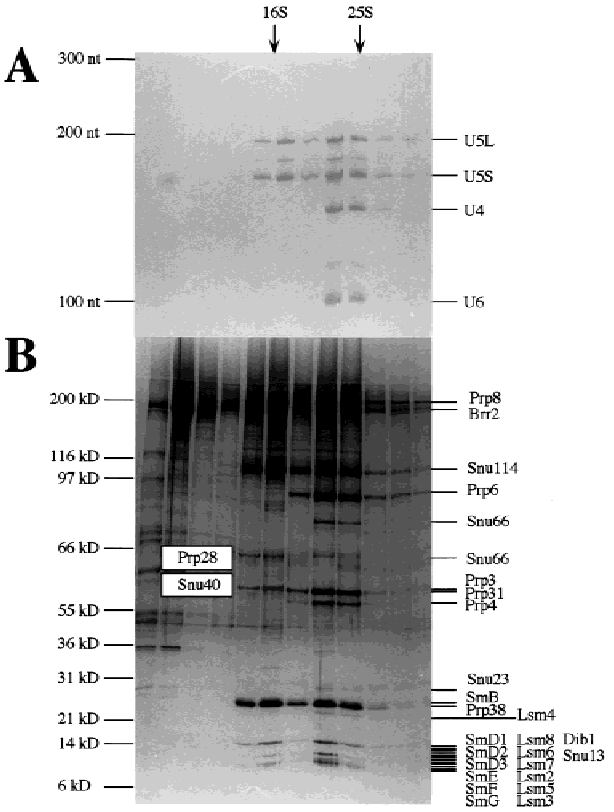


**FIGURE 1.** RNA and protein associated with the Brr2 protein. Affinity purified Brr2-containing material was separated by glycerol gradient sedimentation. **A:** Nucleic acids precipitated from odd fractions of the glycerol gradient were separated by denaturing gel electrophoresis. Locations of the sedimentation of the 16S *Escherichia coli* rRNA and the 25S *S. cerevisiae* rRNA are shown by arrows positioned over the figure. Positions of the yeast U5L, U5S, U4, and U6 snRNAs are shown on the right. Molecular mass markers are shown on the left. **B:** Precipitated proteins from the glycerol gradient fractions were separated by SDS-PAGE. Silver-stained proteins are presented with their identities shown on the right of the figure for tri-snRNP-associated and -shared U5 snRNP associated proteins. U5 proteins not present in the tri-snRNP are labeled in boxes toward the left side of the figure. Note that the lower species of the Snu66 protein is a degradation product (Gottschalk et al., 1999).

## RESULTS

### Purification of the yeast U5 snRNP

The yeast U5 snRNP was purified by affinity chromatography using a strain that possesses a Brr2 protein tagged with a polyoma epitope at its carboxy-terminus. Figure 1 shows the results from the immunopurification and glycerol gradient sedimentation of the Brr2-containing snRNPs. In Figure 1A, silver-stained RNA extracted from every other fraction of a 10–30% glycerol gradient is shown. In fractions corresponding to ~16S, the two isoforms of yeast U5 snRNA are exclusively represented, indicating that the U5 snRNP is a 16S particle in yeast. Fractions corresponding to ~25S indicate that epitope-tagged Brr2 also immunopurifies the 25S U4/U6•U5 snRNP. Proteins copurified with the Brr2-associated snRNPs were precipitated with acetone from the corresponding fractions shown in Figure 1A. In Figure 1B, the U5 and U4/U6•U5 snRNP proteins are shown; excess amounts of Brr2 in the second lane of Figure 1B likely result from the overexpression of the protein due to its presence on a low copy plasmid, rather than single copy in the genome. Preparative amounts of the U5 snRNP fractions were prepared as previously described for the yeast U4/U6•U5 snRNP (Gottschalk et al., 1999; Stevens & Abelson, 1999) and separated by SDS-PAGE electrophoresis. Bands corresponding to the U5 snRNP proteins were

excised and identified by mass spectrometry as described. The identified proteins are listed in Figure 1B and distinguishing features are presented in Table 1.

### Purification of the yeast U6 snRNP

Prp24 was previously reported as a U6 snRNP-associated protein (Shannon & Guthrie, 1991). The genomic open reading frame of Prp24 was modified to include the CHP epitope (Stevens, 2000) by a knock-in procedure. The Prp24 protein is reported to be absent from the U4/U6 snRNP and known to be absent from the U4/U6•U5 snRNP (Gottschalk et al., 1999; Stevens & Abelson, 1999), making it an ideal candidate protein to tag for U6 snRNP purification. Figure 2A shows that, indeed, a U6 snRNA-containing complex that sediments at 7S in the glycerol gradient is purified by polyoma antibody immunochromatography. There is no U4 snRNA anywhere in the gradient as determined by silver staining. The eight U6 snRNA-associated proteins are identified in Figure 2B. All seven of the Sm-Like or LSM proteins are present as well as the epitope tagged Prp24 protein. The proteins identified by mass spectrometry are listed in Table 2.

### Phenotypes of *SNU13*, *SNU23*, *SNU40*, *SNU66*, and *DIB1* genetic disruptions

Pre-mRNA splicing itself is essential in *Saccharomyces cerevisiae* as evidenced by the lethal effects of removing many of the genes coding for proteins involved in pre-mRNA splicing. To test whether these five novel genes were essential, we performed precise gene disruption analysis (Wach et al., 1994). The kanamycin resistance gene (KAN) conferring geneticin resistance in yeast was amplified by polymerase chain reaction (PCR) using primers that contained 50 nt of homology to the sequences upstream and downstream of the start and stop codons of the respective genes. When transformed into a diploid cell, homologous recombination of the sequences surrounding the genes resulted in a replacement of the gene sequence with that of the selectable marker. To ensure that the homologous recombination was at the correct locus, PCR was performed using gene-specific and KAN-specific primers as previously described (Wach et al., 1994). As

**TABLE 1**. Proteins present in the yeast U5 snRNP.

| Yeast protein name | SGD ORF[a] | Calculated MW[b] | No. peptides sequenced | Human homolog |
|---|---|---|---|---|
| Prp8 | YHR165C | 279.5 | 25 | U5-220kD |
| Brr2 | YER172C | 246.1 | 32 | U5-200kD |
| Snu114 | YKL173W | 114 | 12 | U5-116kD |
| Prp28 | YDR243C | 66.6 | 26 | U5-100kD |
| Snu40 | YHR156C | 40.3 | 15 | U5-52K |
| SmB | YER029C | 22.4 | 8 | SmB/B′ |
| SmD1 | YGR074W | 16.2 | 2 | SmD1 |
| Dib1 | YPR082C | 16.7 | 5 | U5-15kD |
| SmD2 | YLR275W | 12.8 | 2 | SmD2 |
| SmD3 | YLR147C | 11.2 | 6 | SmD3 |
| SmE | YOR159C | 10.4 | 2 | SmE |
| SmF | YPR182W | 10 | 2 | SmF |
| SmG | YFL017W-A | 8.5 | 2 | SmG |

[a] *Saccharomyces* Genome Database open reading frame notation.
[b] Calculated relative molecular weight in kilodaltons.

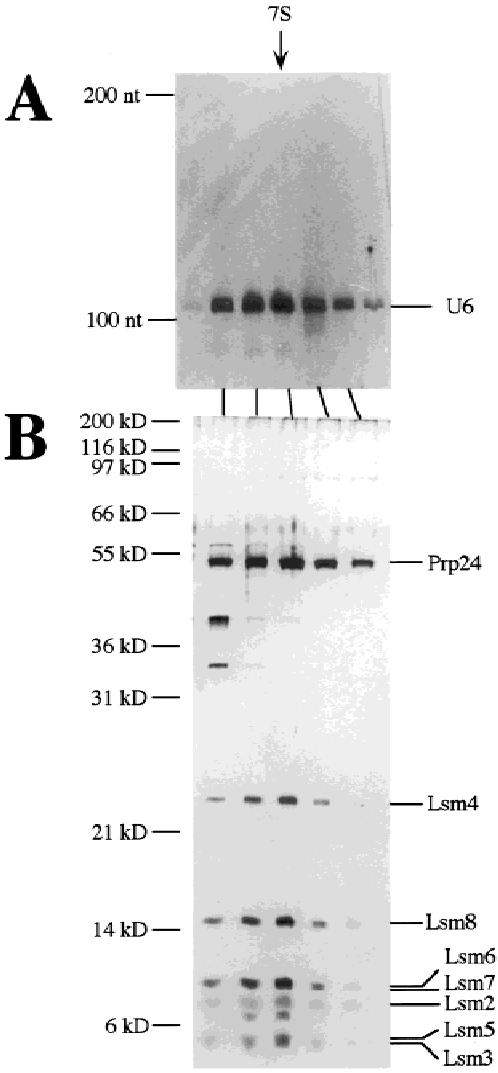


**FIGURE 2.** RNA and protein associated with the Prp24 protein. Affinity purified Prp24-containing material was separated by glycerol gradient sedimentation. **A:** Nucleic acids precipitated from odd fractions of the glycerol gradient were separated by denaturing gel electrophoresis. Location of the sedimentation of the 7S IgG protein is shown by an arrow positioned over the figure. Position of the yeast U6 snRNA is shown on the right. Molecular mass markers are shown on the left. **B:** Precipitated proteins from the glycerol gradient fractions were separated by SDS-PAGE. Silver-stained proteins are presented with their identities shown on the right of the figure. The proteins in the leftmost protein lane migrating at 38 and 32 kDa are glyceraldehyde-3-phosphate dehydrogenase and a degradation product thereof, respectively.

**TABLE 2**. Proteins present in the yeast U6 snRNP.

| Yeast protein name | SGD ORF[a] | Calculated MW[b] | No. peptides sequenced | Human homolog |
|---|---|---|---|---|
| Prp24 | YMR268C | 50.9 | 30 | Unknown |
| Lsm4 | YER112W | 21.2 | 4 | hLsm4 |
| Lsm8 | YJR022W | 14.5 | 8 | hLsm8 |
| Lsm6 | YDR378C | 13.8 | 3 | hLsm6 |
| Lsm7 | YNL147W | 12 | 5 | hLsm7 |
| Lsm2 | YBL026W | 11.1 | 4 | hLsm2 |
| Lsm5 | YER146W | 10.4 | 2 | hLsm5 |
| Lsm3 | YLR438C-A | 10 | 1 | hLsm3 |

[a] *Saccharomyces* Genome Database open reading frame notation.
[b] Calculated relative molecular weight in kilodaltons.

shown in Figure 3A, dissection of the sporulated diploid resulted in the recovery of only two spores for *SNU13*, *SNU23*, and *DIB1*, none of which were KAN$^+$ (data not shown). This indicated that these genes are essential. Disruption of the *SNU66* locus, however, did not result in any nonviable spores at 30 °C, indicating that the *SNU66* gene is not essential. Similarly, disruption of the *SNU40* locus did not result in any nonviable spores at any temperature tested (16 °C, 23 °C, 30 °C, 37 °C; data not shown).

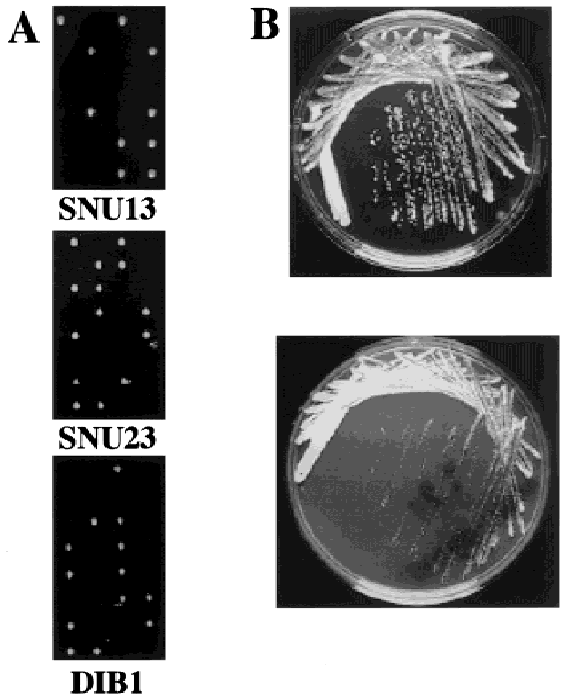


**FIGURE 3.** Disruption phenotypes of the novel tri-snRNP proteins. **A:** Dissected spores from heterozygous diploids from *SNU13/snu13*::*KAN*, *SNU23/snu23*::*KAN*, and *DIB1/dib1*::*KAN* strains as described in the text. All viable spores shown were KAN− indicating there was no growth in the absence of the respective genes. **B:** Growth of heterozygous diploid *SNU66/snu66*::*KAN* strain (top panel) and the *snu66*::*KAN* haploid (bottom panel) at the nonpermissive temperature. Strains were streaked on selective media and grown for 20 days at 16 °C.

## Removal of *SNU66* confers a cold-sensitive phenotype

The *SNU66* gene was shown to be inessential, as KAN$^+$ spores were viable (data not shown). Growth at 23 °C, 30 °C, and 37 °C was similar to wild type for this strain; however, at 16 °C, there was a severe growth defect. Indeed after 20 days growth at 16 °C, only very small colonies were detected, whereas diploid, heterozygous for the *SNU66* deletion grew at a wild-type growth rate (Fig. 3B).

## Genetic depletion of the *SNU13*, *SNU23*, and *DIB1* genes results in an in vivo pre-mRNA splicing defect

Because the *SNU13*, *SNU23*, and *DIB1* genes are all essential, we employed the GAL expression system to determine whether there was an in vivo splicing phenotype upon metabolic depletion of the protein. The open reading frames were cloned into a pRS425-derived vector that contained the entire intergenic region between the *GAL1* and *GAL10* genes. In Figure 4A, the in vivo splicing of the U3A and U3B snoRNA is shown in these strains and their parental strain grown in the permissive galactose-containing medium, and in the repressing glucose-containing medium. After 12 h in the repressing conditions, the GAL-driven strains display a

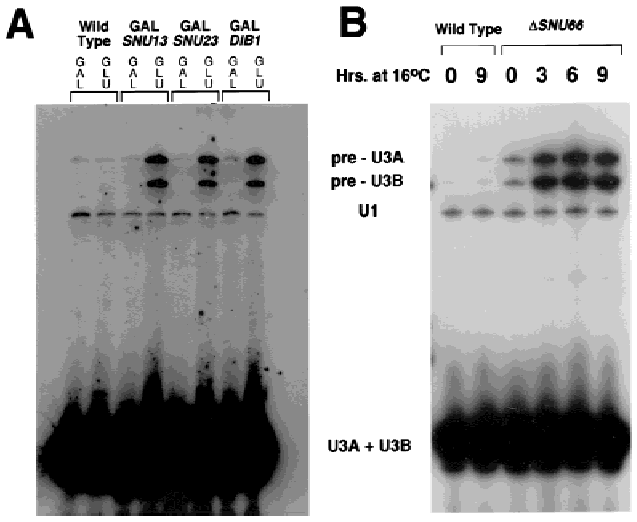


**FIGURE 4.** Splicing defects resulting from the elimination of U4/U6•U5 snRNP proteins. **A:** Genetically depleted Snu13, Snu23, and Dib1 proteins in strains constructed for this purpose were used for assaying the splicing of U3A and U3B snoRNA. **B:** Splicing of the U3A and U3B snoRNAs is severely inhibited in the cold-sensitive Δ*SNU66* haploid strain grown at the nonpermissive temperature. The wild-type parental strain and the Δ*SNU66* strain were grown at 30 °C (time = 0) and shifted to 16 °C for the indicated time periods. Total RNA was harvested and subjected to primer extension as described in the text. A U1 snRNA primer extension product, as a loading control, is also shown. Locations of the U1 and spliced and unspliced U3A and U3B snoRNA primer extension products are represented with text.

pronounced accumulation of the pre-U3A and pre-U3B species. However, in the parental strain, growth in glucose-containing medium had no effect on the splicing of these RNAs.

### Growth of Δ*SNU66* at 16 °C reveals an in vivo pre-mRNA splicing defect

To test the pre-mRNA splicing phenotype of the Δ*SNU66* deletion, a haploid yeast strain containing a disruption of the *SNU66* coding region was grown at room temperature and shifted to 16 °C for increasing lengths of time. Total RNA was harvested from parental strains and Δ*SNU66* strains at the indicated time points indicated in Figure 4B. Pre-mRNA splicing activity was tested in vivo by primer extension of the U3A and U3B snoRNAs. Shifting the temperature of the parental strain had virtually no effect on the accumulation of pre-U3A and pre-U3B. Even when grown at the permissive temperature of 30 °C, the presence of precursors to the mature U3A and U3B indicate a pre-mRNA splicing defect prior to shifting to the nonpermissive temperature. Upon temperature shift, there is a rapid and pronounced accumulation of the pre-U3A and pre-U3B, indicating the cold sensitive phenotype correlates to a severe pre-mRNA splicing defect.

### Dib1 is contained in but does not immunopurify the yeast U5 snRNP

The human U5 and U4/U6•U5 snRNP were previously been shown to contain a 15-kDa protein (Behrens & Lührmann, 1991). Recently, this human protein was identified as a homolog of the yeast Dib1 protein (Reuter et al., 1999). An epitope-tagged version of the Dib1 protein was used for large-scale purification of the Dib1-associated complexes from yeast. Thirty-liter equivalents of yeast culture were used to prepare yeast splicing extract. Epitope-tagged Dib1 containing complexes were purified by polyoma antibody chromatography and the column eluate was layered onto a linear 10–30% glycerol gradient. The RNA extracted from the gradient fractions was analyzed and is presented in Figure 5A. The U4/U6•U5 complex is purified by this procedure, but not the U5 snRNP.

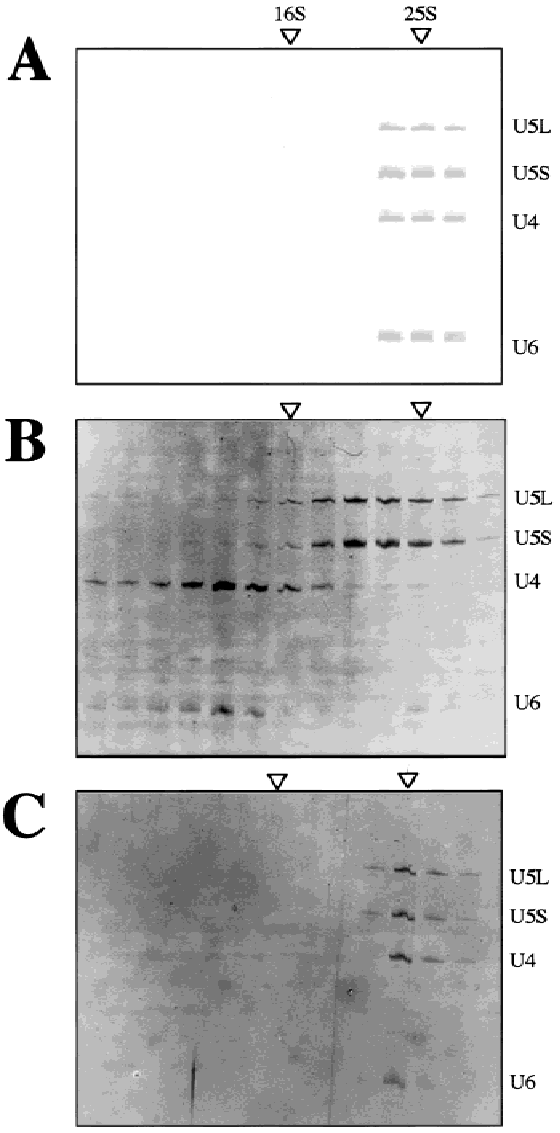


**FIGURE 5.** The yeast U4/U6•U5 snRNP undergoes extensive rearrangements upon addition of ATP or dATP. **A:** Nucleic acids from Dib1 affinity purified and glycerol gradient sedimented yeast U4/U6•U5 snRNP are shown. The 16 and 25S peaks from 16S *E. coli* rRNA and the 25S *S. cerevisiae* rRNA are shown by arrows positioned over the figure. **B:** Nucleic acids from a sample of purified U4/U6•U5 snRNP treated with ATP are shown. **C:** Nucleic acids from a sample of purified U4/U6•U5 snRNP treated with GTP are shown. Positions of the U5L, U5S, U4, and U6 snRNAs are shown on the right side of the figure.

### Purified U4/U6•U5 snRNP disintegrates upon addition of ATP or dATP

As the yeast Brr2 protein contains two ATP helicase-like domains (Noble & Guthrie, 1996) we recapitulated the ATP-driven disintegration of the tri-snRNP demonstrated previously (Raghunathan & Guthrie, 1998) using highly purified tri-snRNP and all eight ribo- and deoxyribo-nucleotides. Extracts from a tagged Dib1 strain were prepared and immunopurified Dib1-containing material was separated by glycerol gradient sedimentation. U4/U6•U5 snRNP-containing peaks were pooled, dialyzed in isotonic buffer containing 8% glycerol, distributed into separate tubes, and incubated at 4 °C for 30 min in the presence of 0.5 mM ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, or dTTP. These reactions were subsequently resedimented on identical 10–30% glycerol gradients as described above. Each gradient was manually fractionated and odd fractions were phenol extracted and nucleic acids were precipitated with ethanol. The silver-stained RNAs from reactions containing ATP and GTP are shown in Figure 5B

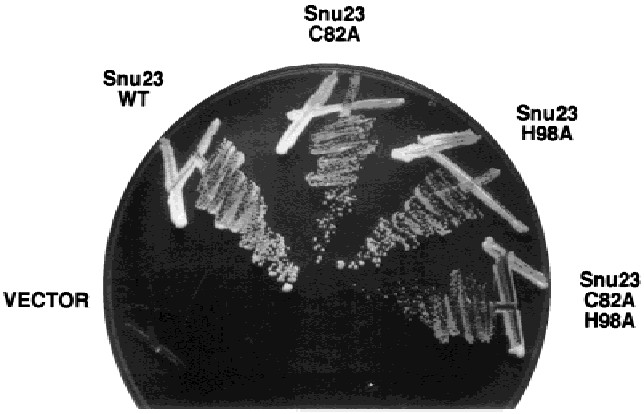


**FIGURE 6.** Effects of mutations in the putative zinc finger of the yeast Snu23 polypeptide. The first cysteine of the $C_2H_2$-type zinc finger (Snu23 C82A), the first histidine (Snu23 H98A), and combination of the two mutations was shuffled into a yeast strain containing a counterselectable *SNU23* gene. Controls include the empty vector and the wild-type *SNU23* gene contained on an identical plasmid.

and C, respectively. The reaction containing dATP was indistinguishable from Figure 5B; reactions containing CTP, UTP, dGTP, dCTP, or dTTP were all similar to Figure 5C. Additionally, we assayed for gross morphological changes of the U5 snRNP upon addition of ATP or GTP. We could not detect any change in the sedimentation behavior of the U5 snRNP upon addition of those nucleotides to immuno- and gradient-purified U5 snRNP based on the comparisons of the migration of U5 snRNA in treated and untreated samples (data not shown).

### The putative zinc finger of Snu23 is inessential

The primary sequence of Snu23 reveals the presence of a potential $C_2H_2$-type zinc finger. In an effort to determine the essentiality of and other possible functions related to the zinc finger, a mutagenesis strategy was employed. Mutating the first cysteine residue of the zinc finger motif to an alanine had no effect on the growth of the resulting strain (Fig. 6). Similarly, the mutation of the first histidine residue of the $C_2H_2$ motif to alanine had no effect on the growth of the strain (Fig. 6). A combination of the two mutations had only a modest effect on colony size, indicating any function of the zinc finger is not required for viability of the cell.

### Snu13, Snu23, and Snu66 have homologs throughout eukarya

Snu13 has remarkable evolutionary conservation as shown by the extensive sequence identity in Figure 7A. The deeply rooted eukaryote *Giardia lamblia* contains a putative homolog of Snu13, indicating that this primitive eukaryote likely has a pre-mRNA splicing capacity. Snu23 has primary sequence homologs in higher eukaryotes that are as similar throughout the entire sequence from human, mouse, fish, worm, and fly with a higher degree of similarity localized to the putative zinc finger motif. The highly conserved cysteines and histidines are indicated above the alignment with asterisks (Fig. 7B). The Snu66 protein sequence is highly similar to the human and murine SART-1 protein at the amino and carboxyl termini and there exists a homolog of Snu66 in *S. pombe* (Fig. 7C).

## DISCUSSION

Relatively little is known about the assembly of snRNPs and splicing complexes from yeast. Recently, progress has been made in the preparative purification of the U1 snRNP (Neubauer et al., 1997; Gottschalk et al., 1998) and U4/U6•U5 snRNP (Gottschalk et al., 1999; Stevens & Abelson, 1999). We have shown the purification of the U5 and U6 snRNPs from yeast and described the associated factors contained in the particles.

The U5 snRNP from yeast contains 13 proteins, 7 shared, canonical Sm proteins and 6 U5-specific proteins. Polypeptides shared in common between the U5 snRNPs from yeast and human (in parentheses) include Prp8 (U5-220kD), Brr2 (U5-200kD), Snu114 (U5-116kD), Prp28 (U5-100kD), Dib1 (U5-15kD), and the SmB (SmB/B′) SmD1, SmD2, SmD3, SmE, SmF, and SmG polypeptides. Previous results suggested that a

**FIGURE 7.** Phylogenetic comparisons of primary sequence homologs of the Snu13, Snu23, and Snu66 proteins of the yeast U4/U6•U5 snRNP. **A:** The sequence homologs of Snu13; (Mg: *Magnaporthe grisea*, Sc: *Saccharomyces cerevisiae*, Ce: *Caenorhabditis elegans*, Pp: *Pristionchus pacificus*, Br: *Brugia malayi*, Hs: *Homo sapiens*, Mm: *Mus musculus*, Rn: *Rattus norvegicus*, Gg: *Gallus gallus*, Bm: *Bombyx morii*, Dm: *Drosophila melanogaster*, Pt: *Populus tremul*a, Gm: *Glycine max*, At: *Arabidopsis thaliana*, Le: *Lycopersicon esculentum*, Os: *Oryza sativa*, Sp: *Schizosaccharomyces pombe*, Dr: *Danio rerio*, Gl: *Giardia lamblia*). **B:** Sequence alignments of putative Snu23 homologs. The conserved cysteines and histidines of the putative zinc finger motif are indicated by asterisks above the sequence alignment. **C:** Sequence alignments of the N-terminus and C-terminus of putative Snu66 homologs. h- and m- SART-1 are the human and murine SART-1 homologs. Note that the amino terminal region of the human, murine, and worm sequences are truncated, as is a central domain of all of the sequences containing very limited homology.

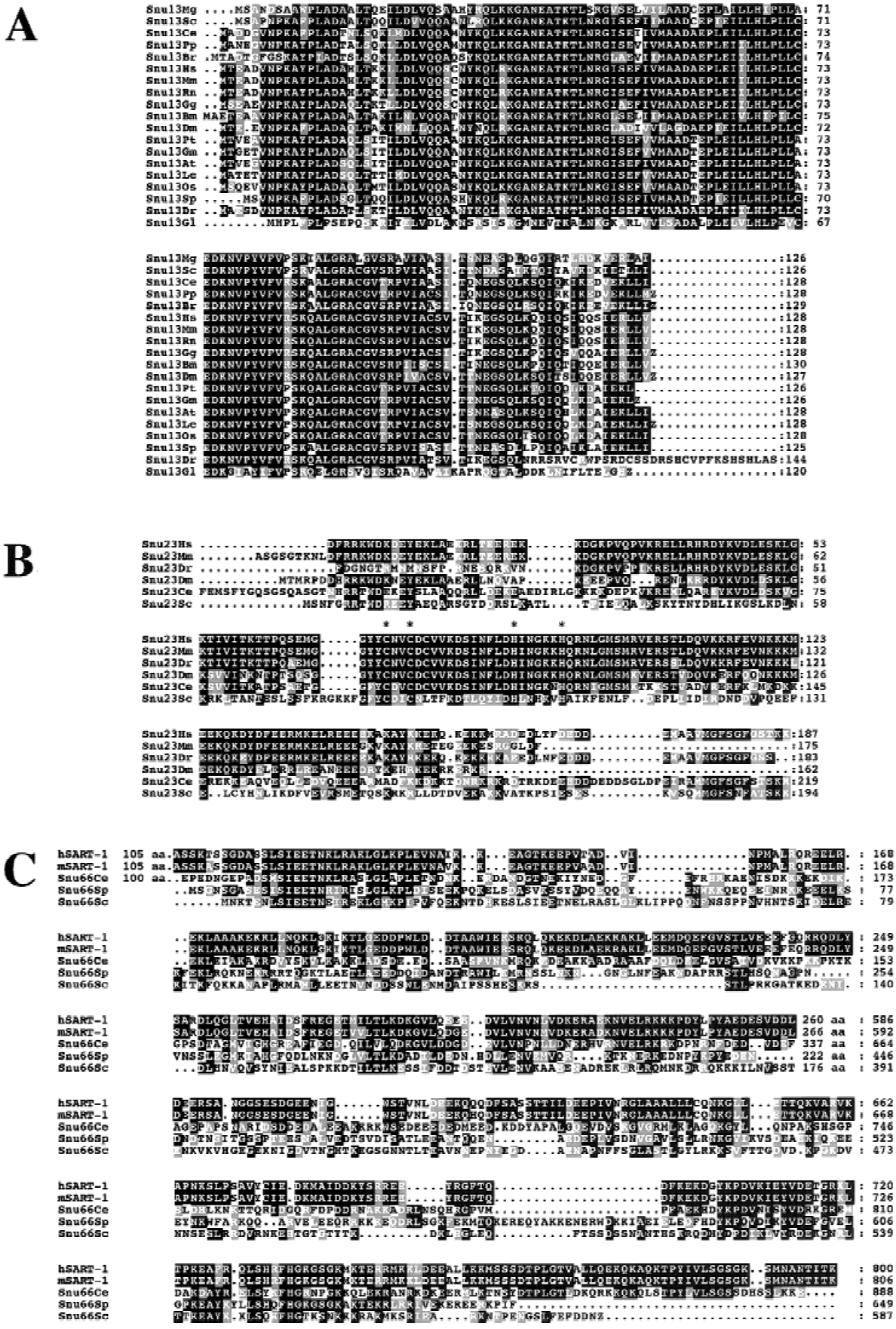


**FIGURE 7.** (*Legend on facing page.*)

partially purified yeast U5 snRNP may not contain the Brr2 protein (Neubauer et al., 1997); however we have now shown that this 16S particle does contain Brr2 (see below). Curiously, the Snu40 gene product has a calculated relative molecular weight of 40 kDa, yet migrates at 56 kDa in SDS-PAGE. The Snu40 gene product is an apparent yeast homolog of the human U5-52K protein (P. Fabrizio, pers. comm.). Proteins associated with the U5 snRNA in human, but not associated with the yeast U5 snRNA, include the human homolog of the yeast Prp6 gene product (Makarov et al., 2000), a human Snu66 homolog (SART-1 or U5-110kD), which is a component of both the yeast and human tri-snRNP but only the human U5 snRNP (Makarova et al., 2001), and the WD-repeat containing 40 kDa protein (U5-40kD; Achsel et al., 1998). It is unlikely that this U5 snRNP is a result of salt-induced decomposition of the yeast tri-snRNP based on two criteria: (1) U4 and U6 snRNAs are only detectable in regions of the glycerol gradient corresponding to the tri-snRNP, and (2) the U5 snRNP contains the Prp28 and Snu40 polypeptides not contained in the U4/U6•U5 snRNP. Lührmann and colleagues have shown that the human Prp28 homolog (U5-100kD) is highly salt labile, but is present in both the human U5 and U4/U6•U5 snRNPs (Behrens & Lührmann, 1991; Teigelkamp et al., 1997). We note that although the U5-snRNP proteins Prp28 and Snu40 migrate coincidentally with different proteins present in the tri-snRNP fractions (Fig. 1B), fractions submitted for MS/MS analysis did not contain peptides from the tri-snRNP and unambiguously identified Prp28 and Snu40. We deduce from our analyses of the yeast U5 and U4/U6•U5 snRNPs that the yeast U4/U6•U5 snRNP may not be a functional particle in vivo, as it does not contain the Prp28 polypeptide. Furthermore, we have identified a particle purified under splicing conditions that contains 85% of all splicing factors and may represent a preassembled spliceosome lacking pre-mRNA (submitted). This would indicate that discrete U1, U2, and U4/U6•U5 snRNPs are, like the U5 and U6 snRNPs, intermediates in a larger complex relating to the assembly of the spliceosome. They may also be viewed as salt-induced decay product of these larger complexes.

In a separate study, Gottschalk et al. (this issue) have isolated a U5 snRNP with a remarkably different composition. Their U5 snRNP was purified initially by virtue of an association with the U1 snRNP. A copurifying polypeptide of ~40 kDa unique to their U5 snRNP was epitope tagged and affinity purified a snRNP lacking Brr2, Prp28, Snu40, and Dib1 indicating that the U5 snRNP exists in different forms in yeast. As the proteins used for tagging in the two snRNPs are mutually exclusive in their respective snRNPs, it is not surprising that there was a difference in the character of the snRNPs. The functional relevance of these different forms is currently unknown.

The U6 snRNP purified in these studies contains eight proteins. Antiserum directed against the Prp24 protein was previously reported to immunoprecipitate U6 snRNA (Shannon & Guthrie, 1991). We used an epitope-tagged Prp24 to immunopurify the yeast U6 snRNP. The remaining seven proteins present in the isolated snRNP were Lsm2, Lsm3, Lsm4, Lsm5, Lsm6, Lsm7, and Lsm8. Séraphin and colleagues have inferred this composition of the U6 snRNP from their purification of all Lsm3-containing or Lsm8-containing snRNPs (Bouveret et al., 2000); however, the purified material they analyzed was a mixture of U6, U4/U6, and U4/U6•U5 snRNPs. We present here the first demonstration of an integral particle containing the above proteins with the U6 snRNA and demonstrate that it is a 7S particle based on its sedimentation in glycerol gradients.

Snu13, Dib1, and Snu66 and their human counterparts are contained in both the yeast and human tri-snRNPs, but the Snu23 polypeptide that is present in the yeast tri-snRNP is not present in the purified human tri-snRNP. Analysis of the tri-snRNP protein genes shows that the *SNU13*, *SNU23*, *and DIB1* genes are essential for vegetative growth of *S. cerevisiae*, and that removal of the *SNU66* gene confers a cold-sensitive growth phenotype. Removal of the *SNU40* gene conferred no growth phenotype at any temperature. The highly conserved Snu13 polypeptide has also been shown recently to be a component of yeast Box C/D small nucleolar RNPs (Watkins et al., 2000), implying that its function may extend to other cellular processes. The Snu23 polypeptide that contains a nonessential putative zinc finger is less highly conserved than Snu13 based on primary sequence homology. Dib1, which was initially identified as an *S. pombe* cell cycle factor (Berry & Gould, 1997) has primary sequence homology to the thioredoxin family of proteins and the crystal structure of the human homolog has recently been determined (Reuter et al., 1999). Although Snu66 is not essential for growth at ambient temperatures, it is required for efficient growth at low temperatures (~16 °C). Although the inessential Snu40 gene product is associated only with the U5 snRNP and not the yeast U4/U6•U5, it is possible that Snu40 plays a minor role in the biogenesis or stability of the yeast U5 snRNP.

We demonstrated that genetic depletion of Snu13, Snu23, and Dib1 as well as the removal of the *SNU66* gene confer splicing defects as judged by the accumulation of precursors to the U3A and U3B snoRNAs that contain pre-mRNA-like introns, confirming their role in the pre-mRNA splicing reaction. While this work was in progress, others have shown a splicing defect conferred by removal of *SNU66* (van Nues & Beggs, 2001), genetic depletion of the Dib1 (Reuter et al., 1999), and Snu23 proteins (Gottschalk et al., 1999) and that antibodies directed against the human homolog of Snu13 inhibit splicing (Nottrott et al., 1999).

We have demonstrated a structural difference in the spatial arrangement of the Dib1 polypeptide contained in the U5 and U4/U6•U5 snRNP. One may posit that the increased complexity of the tri-snRNP would create the greater likelihood of steric hindrance regarding the epitope used for immunopurification; however, the opposite proved true. The Dib1 antigen was accessible in the yeast U4/U6•U5 snRNP, yet was unavailable in the yeast U5 snRNP. This is an indication that in the use of epitope tags, one should probably not exclude the presence of a protein in a complex solely based on its inability to immunopurify that species.

Previously, maintenance of the integrity of the human U4/U6•U5 snRNP in the presence of ATP was demonstrated (Behrens & Lührmann, 1991). We assayed the lability of the tri-snRNP in the presence of NTPs or dNTPs. Unlike the human particle, the yeast tri-snRNP underwent gross morphological changes resulting in a different sedimentation pattern of the snRNAs upon incubation in the presence of ATP or dATP, although no change was noted in the presence of any other NTP or dNTP. Analysis of the proteins comigrating with the snRNAs was impossible due to the presence of many proteins throughout the gradient, indicating a marked change in the character of this particle in the presence of d/ATP. It is likely that there are factors in yeast splicing extract that maintain the integrity of the tri-snRNP in the presence of ATP, allowing the rearrangement only in the proper spatial and temporal context.

Most of the yeast pre-mRNA splicing snRNPs have now been characterized by the methods used in this article. The yeast U1 (Neubauer et al., 1997; Gottschalk et al., 1998), U4/U6•U5 (Gottschalk et al., 1999; Stevens & Abelson, 1999), and now the yeast U5 and U6 snRNPs have been characterized as they exist in yeast extract. A partial purification of the yeast U2 snRNP containing an SF3b component has also been reported (Caspary et al., 1999). A discrete U2 snRNP similar to that of the human 17S U2 snRNP (Behrens et al., 1993) has not been achieved using our methods (unpubl. observations). Indeed the only particles we have successfully purified that contain U2 snRNA and the SF3a and SF3b complexes are in the context of higher-ordered particles (which also include the tri-snRNP or with the U1 and tri-snRNPs) and that exist only in the low-salt conditions compatible with pre-mRNA splicing (submitted). We hypothesize that the familiar discrete U1, U2, U5, U6, U4/U6, and U4/U6•U5 snRNPs represent both intermediates in the assembly of these higher-ordered particles or salt-induced decay products of the higher-ordered particles. We also suggest that the higher-ordered particles represent a more accurate representation of the machinery charged with the splicing of pre-mRNA. The characterization of the components of the spliceosomal snRNPs in all of their incarnations will allow for the future research aimed at understanding how the particles associate and interact throughout the splicing cycle.

## MATERIALS AND METHODS

### Yeast strains and growth, extract preparation, plasmids, and chemicals

Diploid yeast strains JD51 (MAT **a**/$\alpha$, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200, lys2-801/lys2-801, trp1Δ63/trp1Δ63, ura3-52/ura3-52) and MH2 (MAT **a**/$\alpha$, ade2-101/ade2-101, leu2-3,112/leu2-3,112, his3/his3, trp1Δ901/trp1Δ901, ura3-52/ura3-52) were used for gene disruption. Strain ySS231 (MAT **a**/$\alpha$, leu2Δ0/leu2Δ0, his3Δ1/his3Δ1, lys2Δ0/LYS2, met15Δ0/MET15, ura3Δ0/ura3Δ0, snu13::KAN/SNU13) was purchased from Research Genetics (Huntsville, Alabama). ySS231 was transformed with a wild-type copy of *SNU13* in pRS316 (Christianson et al., 1992). This diploid was sporulated and tetrads dissected according to standard methods and yielded ySS256 (MAT **a**, leu2Δ0, his3Δ1, lys2Δ0, ura3Δ0, snu13::KAN + pSNU13-316), which was used in these studies. Disruption of the *SNU23* (JD51), *SNU66* (MH2), and *DIB1* (JD51) genes was achieved through a one-step gene replacement procedure using the kanamycin gene disruption cassette (Wach et al., 1994). Yeast strain PR132 (MAT **a**, ade2, lys2, his3, ura3, leu2, brr2::LEU2, pPR150 (BRR2-py)) was generously provided by Christine Guthrie (University of California–San Francisco). Yeast strain ySS347 (MAT $\alpha$, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, PRP24CHP::LEU2) was constructed by a knock-in procedure (Lafontaine & Tollervey, 1996; Stevens, 2000). Yeast cultures were grown in YPD medium, and KAN-containing strains selected for using YPD + 280 mg/L Geneticin (Life Technologies, Rockville, Maryland) or on SD medium lacking appropriate nutrients. The RS series of yeast plasmid vectors were used for the cloning and manipulation of yeast DNA (Christianson et al., 1992). Yeast splicing extracts were prepared by blending in liquid nitrogen as previously described (Stevens, 2000). All restriction and cloning enzymes were purchased from New England Biolabs (Beverly, Massachusetts); AMV reverse transcriptase was purchased from Promega (Madison, Wisconsin). Chemicals were purchased from Sigma (St. Louis, Missouri) or Fisher (Pittsburgh, Pennsylvania). Wild-type open reading frames were cloned by polymerase chain reaction (PCR) from genomic DNA using oligonucleotide primers 300 bp upstream of the start codon, and 300 bp downstream of the stop codon as previously described (Stevens, 2000). Epitope tagging of the ORFs was achieved using a PCR-mediated tagging strategy. Briefly, one primer 300 bp upstream of the start codon and one incorporating the penultimate codon followed by a restriction site was used to amplify a cassette by PCR that was ligated into the pCHP425 vector.

### snRNP purification and mass spectrometry analysis of snRNP-associated proteins.

U5 snRNP was purified by polyoma-agarose chromatography (Stevens, 2000) using a strain containing a polyoma-tagged *BRR2* gene (the kind gift of Christine Guthrie;

Raghunathan & Guthrie, 1998). U6 snRNP was purified by polyoma-agarose chromatography using a strain containing a C-terminally CHP-tagged *PRP24* gene created by knock-in procedure described previously (Lafontaine & Tollervey, 1996). The plasmid pCHP425 (Stevens, 2000) was used for the generation of the epitope-tagged cassette. Extract preparation and snRNP purification was essentially as previously described. Coomassie-stained proteins in polyacrylamide gel slices were destained, reduced and alkylated, and digested as previously described (Falany et al., 2001). Peptides extracted from the gel were analyzed by LC/MS/MS using a custom-built capillary LC system interfaced to a ThermoFinnigan LCQ quadrupole ion trap mass spectrometer (Davis & Lee, 1998). Fragment ion mass spectra were screened (Moore et al., 2000) and searched against the NCBI nonredundant database using the Sequest program (Eng et al., 1994). Database matches found by Sequest were then manually validated by comparing the actual and predicted spectra. Spectra of peptides from known contaminants such as human keratin and trypsin as well as single matches to nonyeast proteins were ignored.

## Glycerol gradients and RNA analysis

Velocity sedimentation glycerol gradient analysis was performed using linear 10–30% glycerol gradients in 20 mM HEPES, pH 7.9, 200 mM KCl, 1.5 mM $MgCl_2$, 10 mM $\beta$-mercaptoethanol, 1 $\mu$g/mL leupeptin, 1 $\mu$g/mL pepstatin, and 0.01% NP-40. Samples were spun in a Beckman ultracentrifuge SW-41 rotor at 4 °C for 18 or 24 h at 29,000 rpm. Gradients were fractionated from the top and RNA extracted and analyzed as described above. For the preparative purification of Dib1-associated complexes, a 200-L fermenter growth of the polyoma-tagged *DIB1* gene was performed in YPD as previously described. Liquid nitrogen blended extracts were prepared and $\alpha$-polyoma chromatography performed (Stevens, 2000). The antibody eluate was layered on a linear 10–30% glycerol gradient and sedimented at 29,000 rpm in a Beckman SW41 rotor. Twenty eight 400-$\mu$L fractions were collected from the top and phenol extracted. RNA was precipitated from the aqueous phase by addition of NaOAc, pH 5.3, to 0.3 M followed by addition of 3 vol of 100% ethanol. RNA was electrophoresed on a 5% polyacrylamide (19:1) gel containing 8 M urea in 1× Tris-Borate-EDTA buffer. RNA was visualized by silver staining (Blum et al., 1987).

## Cloning and analysis of mutations in *SNU23*

Oligonucleotide mediated PCR was used to introduce the desired mutations in the *SNU23* gene and cloned into the pRS424 vector (Christianson et al., 1992). Mutated genes were sequenced to confirm the presence of the desired mutation and isolates were selected that did not contain additional mutations. Plasmids were transformed into ySS261 (MAT a, leu2-3,112, his3Δ200, lys2-801, trp1Δ63, ura3-52, snu23::KAN + pSNU23-316) and selected for on SD plates lacking tryptophan. The wild-type *SNU23* plasmid was counterselected on SD medium containing 5-fluoroorotic acid (5-FOA; Boeke et al., 1987).

## Construction and analysis of conditional *SNU13*, *SNU23*, *SNU66*, and *DIB1* alleles

Open reading frames and terminator sequences (300 bp downstream of the stop codon) were amplified by PCR and cloned into pGAL-LEU, a pRS425 derived vector with the entire GAL1-GAL10 intergenic region cloned into the polylinker region. Plasmids containing the GAL-inducible promoters were transformed into the appropriate deletion strains and the wild-type plasmids counterselected on medium containing 5-FOA. Maintenance growth of the strains was performed in YP-Gal medium (YP + 2% galactose). To repress the expression of the respective genes, cells were pelleted and resuspended to OD ($A_{600}$) ~ 0.1 in YPD (10 g yeast extract, 20 g Bacto peptone, 10 g dextrose per liter). Growth proceeded for the indicated times and the OD ($A_{600}$) of the cells was maintained below 0.6. For the analysis of *SNU66*, the strain ySS250 (MAT $\alpha$, ade2-101, leu2-3,112, his3, trp1Δ901, ura3-52, snu66::KAN) was grown in YPD to OD ($A_{600}$) ~ 0.1. The flask was then transferred to a shaking incubator precooled to 16 °C and growth was continued for the indicated time increments, always maintaining an OD ($A_{600}$) < 0.6. RNA was prepared by extracting with hot acid phenol/SDS and U3A and U3B splicing defects were assayed as previously described (Madhani & Guthrie, 1992).

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